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Retroviral vector targ ting for gene therapy

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Abstra t

Th majority of gene therapy protocols have used or plan to use retroviral vectors based upon murine leukaemia virus. These vectors are able to infect many different cell typ s, and the retroviral promoter, which is often used to control the expression of a therapeutic gene, is active in a wid range of different cell types. Safe and efficient gen transfer systems, whether based upon retroviruses or other agents, should deliver beneficial genes only to cells that require their therapeutic action, and these genes id ally should be expressed exclusively in such cells. In this paper, strategies for redirecting the infection spectrum of retroviral vectors in order to obtain cell-targeted g ne delivery are discussed. These strategies include the engineering of the retroviral envelope protein, which, together with the availability of its cognate receptor, determines infectivity, and the use of proteins from other enveloped viruses of both retroviral and nonretroviral rigin in the cell lines used to produce retroviral vector virus particles. Expression targeting can be achieved by limiting the expression of therapeutic genes to the cell type(s) of interest using promoters from genes that are normally active in these cells. This approach to targeting is illustrated using promoters from genes expressed in either the liver, the pancreas or the mammary gland as a means to limit gene expression specifically to the c ll types that make up these organs. The successful utilization of new generations of targeted retroviral vectors in the clinic may well pave the way for superior gene delivery systems of the future that seek out their target cell, delivering a therapeutic gene to and expressing it only in such cells.

Key words:
Gene therapy; retroviral vector; gene delivery; targeting; envelope; receptor; infection; pseudotyping; expression; heterologous promoter; tissue specificity; liver; pancreas; mammary gland

Intr duction

The ability to deliver genes of therapeutic benefit to cells is central to the concept of gene therapy. In order to be effective, therapeutic genes must be delivered to the correct cell type and, for reasons of efficiency and safety, it is often a necessity that the therapeutic gene be delivered exclusively to these cells and to no other. The best example of this is when the therapeutic gene chosen encodes a product that is toxic, a strategy that has been proposed and even used for the genetic therapy of cancer in animal models.[1] Absolute targeted delivery of genes requires that a therapeutic gene be transferred only to one particular cell type. This is difficult to achieve regardless of the gene transfer method chosen, and yet the move towards in vivo gene delivery demands that this criterion be fulfilled.

Secondary control can be exerted at the level of regulation of gene expression. Even if genes are delivered to many different cell types, it is not a foregone conclusion that these genes will be expressed in all cell types. Gene expression is controlled by positively (enhancer) and negatively (repressor) acting regulatory elements that are often, though not exclusively, found upstream of the coding sequences. These regulatory elements are binding sites for cellularly encoded factors that modulate transcription initiation and thus the rate of gene expression. Transcription factors may facilitate (enhance) or hinder (suppress or repress) transcription, and their presence or absence in a cell can determine whether or not a gene can be expressed. It should not be forgotten, however, that other determinants can also contribute to this, including accessibility of the regulatory element to its cognate regulatory factor. Thus the inclusion of regulatory elements that ensure expression of a gene in the pertinent cell type and not in other cells represents a strategy for the targeting of therapeutic genes.

Genetically modified viruses (virus vectors) are promising gene transfer tools because they exploit the ability of these extraordinary agents to efficiently deliver genetic information to cells, a property that has been honed and refined by the pressures of evolution. A number of viruses, including the DNA-containing adenovirus and adenoassociated virus, have been modified to carry heterologous genes for research and m re recently gene therapy purposes,[2] but by far the most commonly used virus has been the retrovirus (for a

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review see reference 3). Retroviruses are particularly suited for gene transfer because they integrate a double-stranded form of their genome into the host cell DNA in a stable fashion, ensuring that all daughter cells of a retrovirus-infected cell also carry the viral genetic information.[3] This characteristic is an important consideration for long-term gene therapy, such as for the treatment of inborn errors, where retroviral vectors are often used, since this property is retained by these vectors (for a more complete overview of retroviral vectors see references 4 and 5). The integration of the retroviral genome into host DNA is performed by a virus-encoded enzyme (integrase), which ensures that integration is an efficient and ordered process,[6] preventing the disruption of the viral genome (or the therapeutic gene that is transferred).

The majority of retrovirus vectors have been constructed using a mouse retrovirus, murine leukaemia virus (MLV), as the basis of the vector system.[4,5] This retrovirus can infect many different cell types, and variants that can infect human cells are already in use for clinical gene therapy. The infection spectrum of retroviruses (and thus derived vector systems) is determined by the interaction between the viral envelope protein and receptor proteins located in the host cell membrane.[7] The wide infection spectrum of MLV suggested that the cellular receptor for this virus is a common cell membrane protein, and this was verified by the recent identification of this protein as a phosphate transporter.[8,9]

Redirection of the infection event

Targeting the infection event of MLV-based retroviral vectors requires that the infection event be redirected. This requires the modification or alteration of the MLV envelope proteins so that they interact with more exclusive cell receptors. A number of strategies, each with differing degrees of success, have been employed to achieve this goal.[5] Direct modification of the viral envelope protein is not as simple as it seems, because of the relatively complex synthesis and processing of this protein, which ensures both its functionality and its ability to become incorporated into newly synthesized virus particles. These requirements place constraints on the replacement or the modification of the envelope protein, since the conformations of certain domains are likely to be critical. Recently the epitopes that interact with the receptor have been mapped to the amino terminus of the virus envelope protein (Figure 1).[10–12] This region has been replaced with

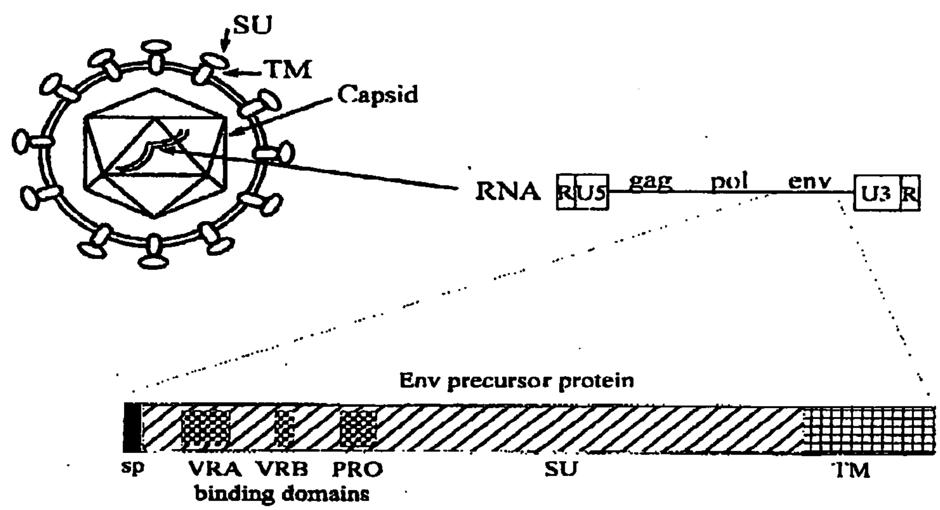

Figure 1
Schematic diagram of the retrovirus particle, genome and envelope protein. Retroviruses are enveloped viruses carrying two copies of a single positive-sense genomic RNA. The genome carries terminal redundancies (R), unique 5' (U5) and 3' (U3) sequences (which later, in the DNA or proviral form of the retrovirus, form the LTRs), as well as three coding regions that encode core (capsid) proteins (gag), the viral reverse transcriptase and integrase enzymes (pol) and the envelope proteins (env). The envelope proteins form the anchor (TM = transmembrane) and protrusions (SU = surface) that extend outwards from the host derived plasma membrane (stalks and ovals). The two envelope proteins (SU and TM) are synthesized as a polyprotein precursor, shown as the enlarged box. A signal peptide (sp) is located at the amino-terminal end of this precursor. The amino-terminal end of the SU protein has been shown to interact with host cell receptors, and may be exchanged for ligand domains that recognize other cellular receptors. The variable region A (VRA) and variable region B (VRB) binding domains have been shown to be involved in the interaction with the ecotropic and amphotropic receptors, whilst the proline-rich region (PRO) is additionally involved in binding to the xenotropic and polytropic receptors.[10]

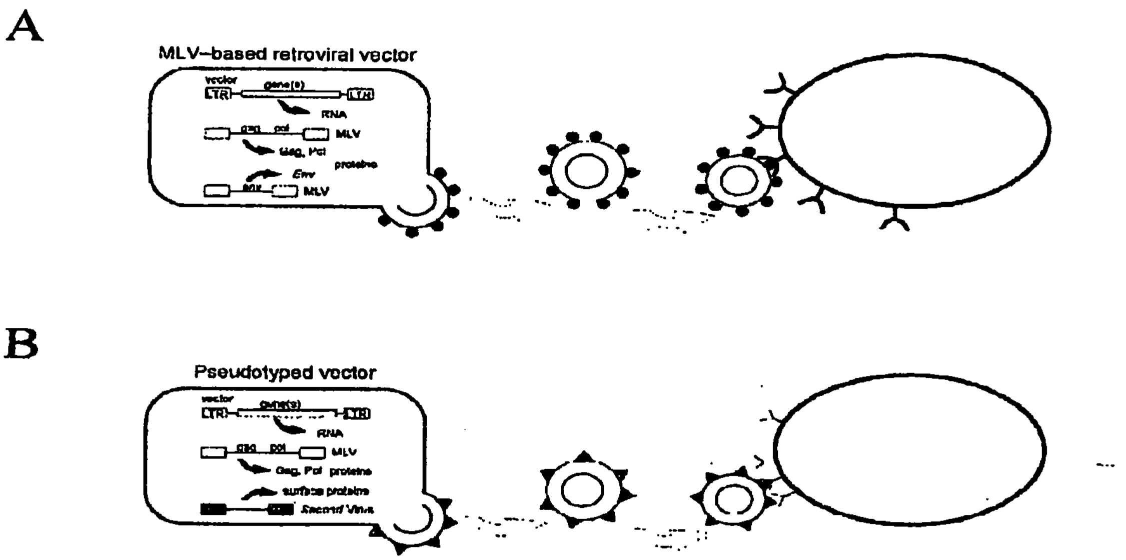

Figure 2
The principle of pseudotyped vectors.
(A) MLV-based retroviral vectors usually comprise three components: a vector construct in which the genes to be transferred are cloned between the two retroviral LTRs, and two other constructs, from which the retroviral gag, pol and env genes are expressed. The RNA transcribed from the vector construct is packaged into the proteins translated from the gag, pol and env constructs. A vector with an infection specificity determined by the Env proteins (●) then buds from the cell, and is able to infect target cells carrying receptors able to interact with the Env protein.
(B) Pseudotyped vectors are essentially the same as standard MLV-based retroviral vectors; however, the env protein is now substituted either by the Env protein of another retrovirus with a different infection specificity (▲) or by the surface proteins of another enveloped virus with a different infection specificity (▲). These pseudotyped viruses now infect a different set of target cells, which carry receptors able to interact with this heterologous surface protein.

epitopes from other proteins that are known to interact with other defined receptors such as erythropoeitin,[13] heregulin[14] or integrin,[15] or with single-chain variable regions of antibodies directed against known epitopes.[16–19] Although promising, the titres that are achieved are compromised by this kind of strategy, and clearly we need to know more about ligand–receptor interactions before retroviral envelope proteins can be modified or even replaced. Nevertheless, these studies provide encouragement to pursue this kind of approach.

Recently, Steven Russell and colleagues have described a new two-step strategy to target retroviral vector receptor binding. The viral envelope protein is modified by the linear addition of a protease cleavage site and the selected receptor ligand domain. After binding the appropriate receptor on the target cell, the receptor/ligand can be cleaved off by expression of the protease. The retrovirus then attaches to its usual receptor on the same target cell, allowing the retrovirus to enter the cell by the natural route, circumventing the entry problems that often occur when retroviruses are targeted to use nonretroviral receptors.[20] Titres of up to 10^6 cfu/ml have been achieved using the EGF-binding domain as a means to target in this two-step system.[21]

Retroviruses ar enveloped viruses, and share with other enveloped viruses the ability to form hybrid virions, for example after co-infection of cells or after superinfection. These mixed virus particles consist of the core and genome of one virus and an envelope carrying the envelope proteins of both viruses. Pseudotyped retroviral vectors have been produced that usually carry the genome and core proteins of MLV and the envelope proteins only of the second virus (Figure 2) (for a review see reference 22). The rhabdovirus, vesicular stomatitis virus (VSV), envelope has been used in this way to generate pseudotyped retroviral vector particles that have greater stability than MLV-based vector virions and are consequently easier to purify. A second advantage of this system is that increased titre of vector virus is produced.[23] The pseudotyped virions display the infection spectrum of VSV, with an incredibly broad host and cell range.[24] While this is useful for some purposes (for example the infection of rare haematopoietic stem cells in a mixtur of many more differentiated cell types), it is highly undesirable with respect to targeted gene delivery. Another problem with the VSV envelope is that it causes cell fusion (Figure 3). Pseudotyped retroviral vectors have been constructed using envelopes of other retroviruses that are l ss promiscuous in their infection spectrum than MLV (reviewed in reference 3). Mouse

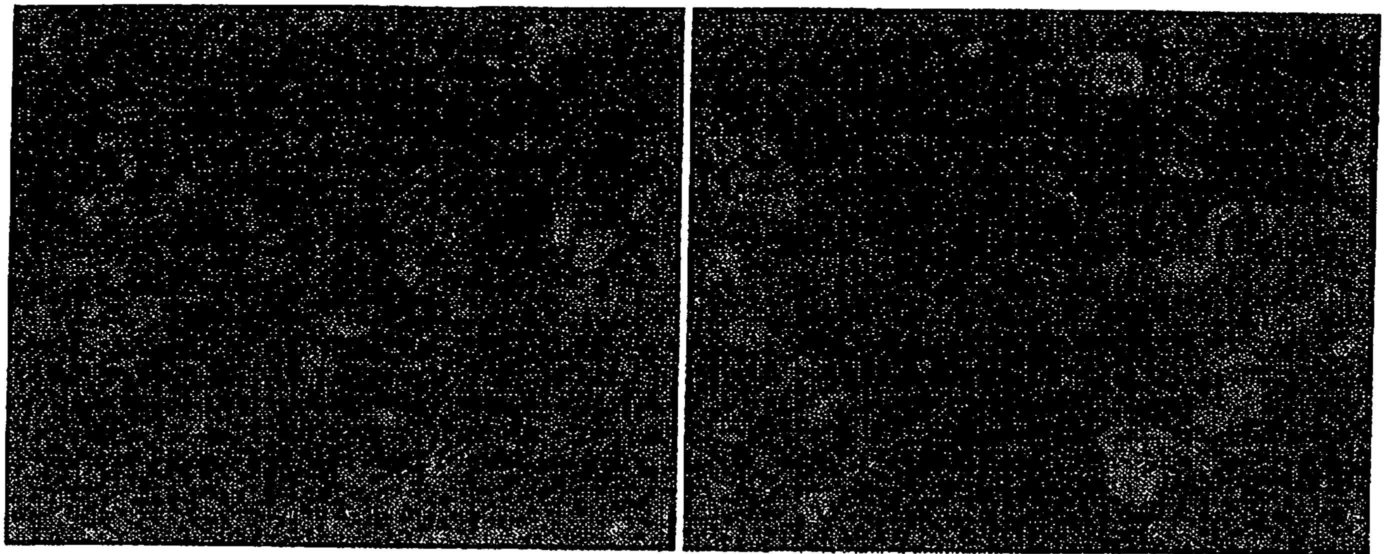

Figure 3
Fusion of cells induced by the VSV G protein.

mammary tumour virus (MMTV) infects predominantly mammary epithelial cells and B lymphocytes. Pseudotyping of an MLV-based retroviral vector with the envelope of MMTV has been shown to permit the vector to infect MMTV permissive cells in cell culture.[25]

Limiting the expression spectrum of th rap utic genes

The MLV promoter is carried within the long terminal repeat (LTR) region of the viral DNA (Figure 1). This promoter can be used to express heterologous genes in retroviral vectors, but it is prone to host-cell-mediated shutdown,[26,27] and, more importantly from the point of view of expression targeting, the MLV promoter is active in many cell types.[28] Heterologous promoters can be included in retroviral vectors to drive the expression of introduced genes in such a way as to leave the MLV promoter intact or so that the retroviral promoter is replaced by the heterologous promoter (Figure 4). Promoters from genes known to be expressed in particular cell types may be useful for targeting the expression of a therapeutic gene to that cell type. One prerequisite for the use of such promoters is that they are molecularly cloned and that the regulatory elements conferring cell-type-specific expression are present in the cloned promoter fragment. A range of promoters from genes that show tissue-specific expression have been used to limit the expression of retrovirally transmitted genes to a number of organs.

The liver

This organ is of interest for gene therapy since it is affected by a number of inherited metabolic disorders, usually resulting from either a loss of or a reduction in enzyme function.[29,30] Also, cancer of the liver is an ideal candidate for gene therapy since it is difficult to treat using conventional therapies.[31] A number of promoters known to be specifically active in hepatocytes have been used in retroviral vectors to target expression of either therapeutic or marker genes to this organ, including those of the α-fetoprotein and phosphoenolpyruvate carboxylase (PEPCK) genes (for a review see reference 5). The efficacy of some of these promoters have been compared for their ability to direct gene expression after in vivo infection of hepatocytes. The human α_1-anti-trypsin promoter was the strongest of the promoters tested in this study, whereas the PEPCK proved a weaker promoter than that of MLV.[32]

The pancreas

This organ harbours both endocrine and exocrine cells. The endocrine cells normally produce insulin, which is required for the effective regulation of blood sugar levels (for a review see reference 33). However, these cells can either lose this ability or become ablated, and the patient must regularly receive insulin. In contrast, pancreatic cancer arises mainly in the exocrine cells of this organ (reviewed in reference 34). These cells normally synthesize digestive enzymes and bicarbonate. Although this cancer is not one of the most common, it has a five-year survival rate of 2%, with a median survival of 20 months.[35]

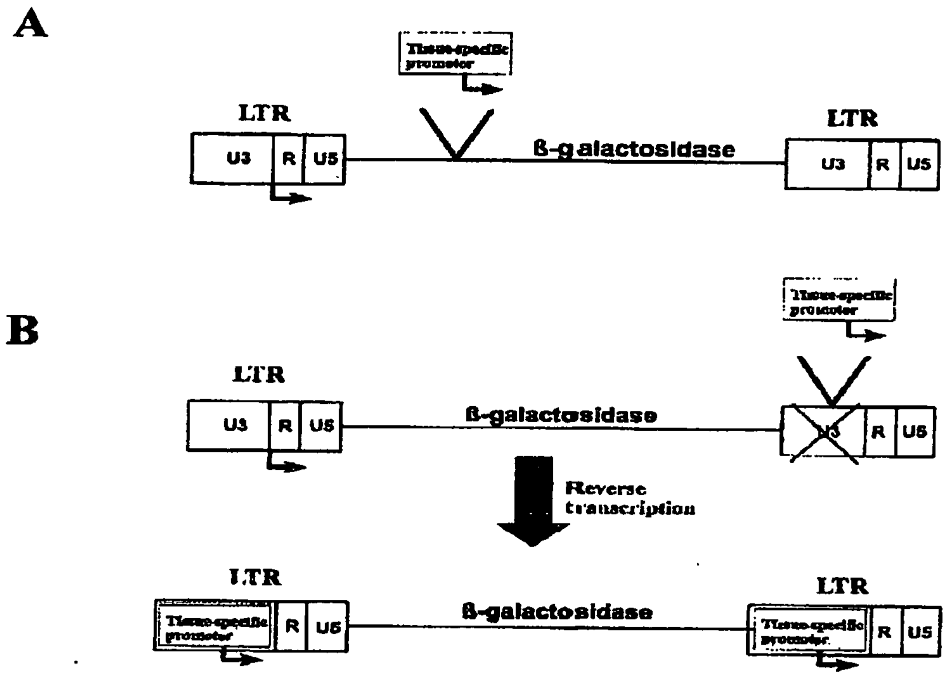

Figure 4
Tissue-specific promoters in retroviral vectors. The figure shows two types of retroviral vector carrying a β-galactosidase indicator gene under the transcriptional control of a tissue-specific promoter. Promoters that are known to direct gene expression to particular cell types can be introduced into retroviral vectors directly upstream of the indicator gene (A). Alternatively, the promoter can be inserted into the U3 region of the long terminal repeat (LTR). As a consequence of the packaging of retroviral genomic RNA, infection of target cells and reverse transcription into a double-stranded DNA proviral form, the inserted promoter becomes duplicated and placed at either end of the viral DNA. Thus the viral promoter, located in the original U3 region, is replaced by the tissue-specific promoter (B).

The glucokinase gene is expressed in the endocrine cell of the pancreas.[36] A 1 kb fragment of the upstream glucokinase promoter[33] was coupled to the coding sequences of the luciferase gene. The resultant construct was transfected into various cell lines and analysed for its ability to direct luciferase expression. Luciferase activity was only detected in the two endocrine-derived cell lines RIN-5 and HIT-T15, and not in exocrine cells or in mammary or kidney epithelial cells (Figure 5A). Further, this promoter gave better expression levels than the relatively strong simian virus 40 (SV40) promoter (Figure 5A). Thus the data suggests that the 1 kb glucokinase promoter fragment carries regulatory elements that direct gene expression to endocrine cells of the pancreas. This promoter fragment is presently being introduced into an MLV-based retroviral vector in place of the viral promoter.

The carbonic anhydrase (CA-II) gene is expressed in endocrine cells of the pancreas.[37] The promoter region (1 kb) of this gene was also coupled to a luciferase gene and introduced into various cells. The CA-II promoter gave rise to good levels of luciferase expression (i.e. comparable to the SV40 promoter) in two exocrine-derived cell lines (Figure 5B). However, the regulatory elements contained in this promoter fragment are not absolutely exocrine-cell-specific, since significant luciferase activity could be detected in kidney cells (CK) and in one of the endocrine-derived cell lines (HIT-15). These data suggest that this promoter fragment either does not contain exocrine-specific regulatory elements (they may be present further upstream) or contains regulatory elements that permit a more generalized expression that overrides exocrine-specific elements to some extent.

The mammary gland

Mammary-gland-specific targeting of the expression of genes encoding toxic products would be useful for the treatment of breast cancer. A number of genes are expressed specifically in the mammary gland, notably those encoding milk proteins, some of which may be expressed in mammary tumours. The whey acidic protein (WAP) is a major component of rodent milk, but the regulatory mechanisms that confer mammary-specific expression upon this gene are present in other mammals,[38] presumably indicating that milk protein genes are

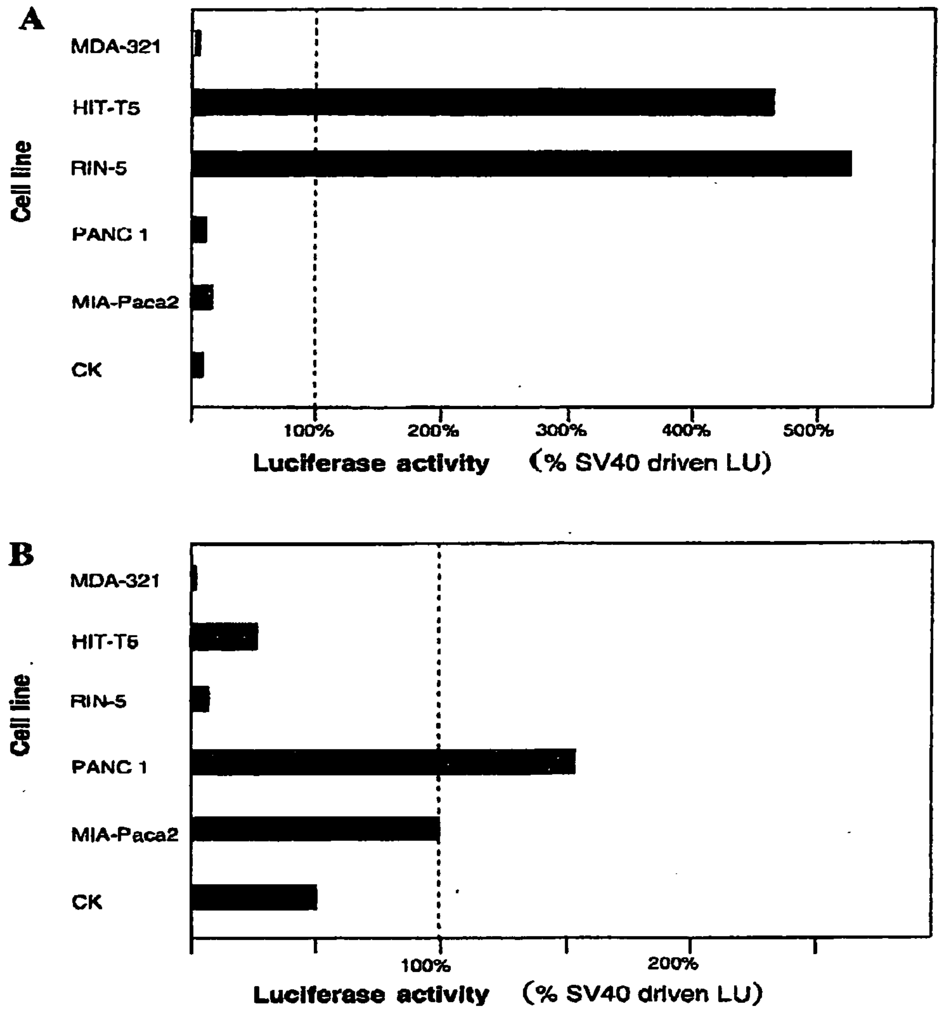

Figure 5
Analysis of putative pancreas-specific promoters. DNA fragments from the endocrine-specific glucokinase (GK) promoter (A) and exocrine-specific carbonic anhydrase II (CAII) promoter (B) were linked to promoterless luciferase indicator genes, and transfected into mammary-tumour-derived MDA-MB-321 cells, HIT-T5 and RIN-5 cells derived from pancreatic endocrine cells, PANC 1 and MIA-Paca2 cells derived from pancreatic exocrine cells, and the kidney-derived cell line CK. Luciferase activity was assayed 48 h post-transfection, and is shown relative to the activity obtained after transfection of a luciferase gene coupled to the SV40 promoter, used as a control (dotted line, 100%).

regulated by common mechanisms. A DNA fragment (2.4 kb) containing the WAP promoter has been shown to direct the expression of a number of genes to the mammary gland in studies using transgenic animals.[39,40] Deletion mutation analysis has suggested that this fragment carries a regulatory element that suppresses WAP expression in all nonmammary cell types owing to the presence of a factor that binds to this region.[41,42] This element has been included in a retroviral vector based on MLV, and initial studies suggest that it directs mammary-specific expression.[25,43]

The erbB2 gene is frequently overexpressed in a number of cancers, including breast[44] and pancreatic[45] cancers, as a result of increased transcription. This is mediated by a binding site for the novel transcription factor OB2-1.[46] The erbB2 promoter has been used to target the expression of a suicide gene, cytosine deaminase, to tumour cells that overexpress erbB2, using a retroviral vector. Cytosine deaminase converts the nontoxic prodrug 5-fluorocytosine (5-FC) to the toxic metabolite 5-fluorouracil. Breast- or pancreatic-derived tumour cell lines overexpressing erbB2 showed reduced

cell survival after treatment with 5-FC compared with those that did not overexpress erbB2.[47]

Futur perspectives

The judicious combination of strategies aimed at targeting the infection spectrum of viral vectors with those limiting expression of transmitted therapeutic genes will form a two-tier restriction system for the specific delivery of therapeutic gene products. The principles discussed above for retroviruses can equally be applied to other viral vectors. Recently it has been reported that the infection spectrum of adenovirus v ctors can be altered by replacement of the penton base RGD motif with a motif specific for another receptor.[48] Similarly, the expression of genes carried in adenoviral vectors has been shown to be targetable by incorporating cellular promoters within the adenoviral genome.[49] Although much work has still to be done to optimize and refine these targeting systems, the points raised have been set and the principles proved, suggesting that in the near future, high-efficiency, targeted therapeutic gene transfer systems will be on hand.

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